

Method of testing antibiotic sensitivity of spirochaetes, using antibiotic discs

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A rapid method of evaluating the susceptibility of spirochaetes to antibiotics would be convenient for investigators. Schneierson (1954) described a method of testing the susceptibility of strains of potentially pathogenic species, in which discs containing standard amounts of antibiotics were dropped into broth, and the broth was inoculated with the organism under study. He found that the results compared favourably with those of the tube-dilution method. Antibiotic concentrations were determined by dividing the disc concentration by the volume of the broth.

The purpose of this investigation was to develop a rapid method for determining the susceptibility of representative strains of treponemes using antibiotic discs.

Materials and methods

The treponeme strains used in this study and their sources were described in a previous communication (Abramson and Smibert, 1971). The strains were *Treponema phagedenis* biotype Reiter and Kazan; *T. refringens* strains refringens, calligyrum, and Nichols; *T. denticola* strains microdentium FM, ambiguum, and T-32-A; *T. vincentii* N-9; rumen fluid requiring strains from the human oral cavity HO-5, HO-18, and HO-27; rumen fluid requiring strains from pig faeces PF-23, PF-31, PF-39, PF-28, and PF-44. Two free-living spirochaetes of the genus *Spirochaeta* (DP-1 and DP-2), isolated from muddy duck-pond water, were also included in this study.

MEDIA

Pre-reduced anaerobically sterilized media were prepared as described in "Outline of Clinical Methods in Anaerobic Bacteriology" (Anaerobe Laboratory, 1970). Inoculations were carried out under O₂-free nitrogen using the VPI Anaerobic Culture System*.

The basic medium for treponemes requiring serum (PYG medium) contained peptone M† 2 g.; yeast

extract‡ 1 g.; dextrose 1 g.; agar 0.2 g.; ammonium sulphate 0.05 g.; soluble starch‡ 0.05 g.; TEM-4T§ 0.016 g.; L-cysteine HCl hydrate 0.16 g.; sodium bicarbonate 0.5 g.; resazurin solution (25 mg. per cent.) 0.4 ml.; salt solution 50 ml. (anhydrous MgSO₄ 0.02 g.; CaCl₂·2H₂O 0.02 g.; K₂HPO₄ 0.1 g.; KHPO₄ 0.1 g.; NaCl 0.2 g. dissolved in 100 ml. distilled water); distilled water 50 ml. The pH was adjusted to 6.5 before autoclaving and was 6.8 to 7.4 after sterilization.

For cultivation of strains of *Treponema phagedenis* and *T. refringens*, inactivated rabbit serum (12 per cent.) was added aseptically to autoclaved PYG medium. For strains of *T. denticola* and *T. vincentii* cocarboxylase (0.00012 per cent.) was added in addition to the serum.

Rumen fluid-requiring treponemes were maintained in E-medium, which consisted of peptone M 0.05 g.; yeast extract 0.05 g.; dextrose 0.14 g.; agar 0.2 g.; ammonium sulphate 0.05 g.; soluble starch 0.05 g.; L-cysteine HCl hydrate 0.16 g.; sodium bicarbonate 0.5 g.; resazurin solution 0.4 ml.; salt solution 50 ml.; rumen fluid 28 ml.; distilled water 22 ml. The pH was adjusted to 6.5 before sterilizing and was 6.8 to 7.4 after autoclaving. Rumen fluid-requiring treponemes (pig intestinal (PF) isolates and human oral (HO) isolates) were tested in HO medium, which consisted of peptone M 0.1 g.; yeast extract 0.1 g.; dextrose 0.5 g.; agar 0.2 g.; ammonium sulphate 0.05 g.; soluble starch 0.05 g.; L-cysteine HCl hydrate 0.16 g.; sodium bicarbonate 0.5 g.; resazurin solution 0.4 ml.; salt solution 50 ml.; rumen fluid 28 ml.; distilled water 22 ml. The pH was adjusted to 6.5 before autoclaving and was 6.8 to 7.4 after autoclaving.

All tests with serum and rumen-fluid requiring treponemes were done in 7.2 ml. of medium and inoculated with 0.3 ml. of a 24 to 36-hr culture.

Free-living spirochaetes of the genus *Spirochaeta* were cultivated in DP medium which was dispensed in 3.8-ml. volumes in screw-capped tubes and inoculated with 0.2 ml. inoculum. The DP medium contained peptone M 0.1 g.; yeast extract 0.1 g.; dextrose 0.2 g.; agar 0.2 g.; ammonium sulphate 0.05 g.; soluble starch 0.05 g.; L-cysteine HCl hydrate 0.16 g.; sodium bicarbonate 0.5 g.; resazurin solution 0.4 ml. (25 mg. per cent.); salt solution 50 ml.; distilled water 50 ml.

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§Diacetyl tartaric acid ester of tallow monoglycerides, Witco Chemical Co., 277 Park Avenue, New York, New York, 10017 U.S.A.

ANTIBIOTIC DISCS ON AGAR MEDIUM IN BOTTLES

20 ml. of pre-reduced PYG medium containing 0.8 per cent. Ion Agar No. 2* and 10 to 12 per cent. rabbit serum were placed under O₂-free N₂ in rubber-stoppered 4-oz. prescription bottles. Some bottles containing molten serum medium (45° C.) were inoculated with 1 ml. of a 24-hr culture of the Reiter strain of *T. phagedenis*, swirled gently, and slanted. Others were first slanted and the surface inoculated with 1 ml. of the culture. Excess inoculum was removed after 1 hour with a Pasteur pipette. The following antibiotic discs were then aseptically placed on the surface of the medium while the bottles were gassed with O₂-free N₂; penicillin 10 units; polymyxin 300 units; tetracycline 30 µg.; cephalothin, 30 µg. Observations for growth and zones of inhibition were made daily.

ANTIBIOTIC DISCS ON AGAR MEDIUM IN PETRI DISHES

Flasks of molten PYG serum agar were inoculated with the Reiter culture of *T. phagedenis* (1 ml./10 ml. medium), swirled gently, and poured into Petri dishes. Other Petri dishes containing solid PYG serum agar were flooded with 1 ml. of the culture and excess inoculum was removed with a Pasteur pipette. Antibiotic discs (penicillin 10 units; polymyxin 300 units; tetracycline 30 µg.; cephalothin, 30 µg.) were aseptically placed on the surface of the agar and all plates were placed in Brewer jars which were evacuated and filled with O₂-free N₂ twice and finally with 90 per cent. H₂ and 10 per cent. CO₂. Plates in the Brewer jars were incubated for 5 days. Agar plugs were cut from various areas of the agar surface from both bottles and Petri dishes, placed on slides, and observed under darkfield microscopy. Agar plugs were also inoculated into PYG-serum medium.

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ANTIBIOTIC DISCS IN SEMI-SOLID MEDIUM

One antibiotic disc of each concentration was dropped into pre-reduced PYG medium, HO medium, or DP medium. All tubes were inoculated with 0.3 ml. of a 24-hr culture 30 min. after placing the antibiotic disc into the medium. Antibiotic concentrations were calculated by dividing the disc concentration by the total volume of the medium. Observations for growth were made daily for 3 days.

GROWTH INHIBITION

The growth inhibitory concentration of an anti-microbial agent was considered to be the lowest concentration at which growth was inhibited after 3 days' incubation.

Results

Growth of all treponemes on PYG agar medium in prescription bottles was transparent and appeared as a thin film on the surface of the agar, making observations of zones of inhibition difficult. Motile treponemes were seen with darkfield microscopy from agar-plug samples from areas both near antibiotic discs and some distance from the discs. Cultures grew from agar plugs inoculated into PYG-serum medium. No growth was seen on any PYG-serum agar in Petri dishes that were incubated in anaerobic jars and treponemes were not observed under darkfield microscopy from agar plugs cut out from various places around the Petri dishes.

Tables I to IV show the concentrations of anti-microbial agents that inhibited the growth of the treponemes and spirochaetes studied using antibiotic discs in semi-solid media.

TABLE I *Growth inhibition of treponemes by the penicillins using the tube-disc method*

Treponeme strain	Inhibitory concentrations of antimicrobial agents (µg. or units/ml.)							
	Pen ^a	Amp ^e	Clox ^f	Meth ^g	Naf ^h	Ox ⁱ	Phen ^j	Ceph ^k
Reiter ^a	0.3*	0.3	+(0.15)	0.7	+(0.1)	+(0.1)	0.4	4
Kazan ^a	0.3	1	+(0.15)	0.7	+(0.1)	+(0.1)	0.4	4
<i>T. refringens</i>	0.3	0.3	0.15	0.7	0.1	0.1	0.4	4
Calligyrum ^b	0.3	0.3	0.15	0.7	0.1	+(0.1)	0.4	4
Nichols ^b	0.3	0.3	0.15	0.7	+(0.1)	0.1	0.4	4
Microdentium FM ^c	0.3	0.3	0.15	0.7	0.1	+(0.1)	0.4	4
Ambiguum ^c	0.3	0.3	0.15	0.7	0.1	+(0.1)	0.4	4
T-32-A ^c	0.3	0.3	0.15	0.7	0.1	+(0.1)	0.4	4
<i>T. vincentii</i> N-9	0.3	1	0.15	0.7	+(0.1)	+(0.1)	0.4	4
HO 5	+(1)	+(1)	+(0.15)	+(0.7)	+(0.1)	+(0.1)	0.4	4
HO 18	+(1)	+(1)	+(0.15)	+(0.7)	+(0.1)	+(0.1)	0.4	4
HO 27	+(1)	+(1)	+(0.15)	+(0.7)	+(0.1)	+(0.1)	0.4	4
PF 23	0.3	0.3	+(0.15)	0.7	+(0.1)	+(0.1)	0.4	4
PF 31	+(1)	+(1)	+(0.15)	+(0.7)	+(0.1)	+(0.1)	0.4	4
PF 39	0.7	0.3	+(0.15)	+(0.7)	+(0.1)	+(0.1)	0.4	4
PF 28	0.3	1	+(0.15)	+(0.7)	+(0.1)	+(0.1)	0.4	4
PF 44	0.3	1	+(0.15)	+(0.7)	+(0.1)	+(0.1)	0.4	4
DP-1	0.5	0.5	0.25	1.25	+(0.25)	0.25	+(1.5)	7.5
DP-2	+(2.5)	+(2.5)	+(0.5)	+(1.25)	+(0.25)	+(0.5)	+(1.5)	7.5

^a*T. phagedenis*, ^b*T. refringens*, ^c*T. denticola*, ^dK penicillin G, ^eampicillin, ^fcloxacillin, ^gmethicillin, ^hnafticillin, ⁱoxacillin, ^jphenicillin, ^kcephalothin, *concentration at which no growth appeared in 3 days, + = growth at concentration in parentheses

TABLE II Growth inhibition of treponemes by macrolides and other antibiotics using the tube-disc method

Treponeme strains	Inhibitory concentrations of antimicrobial agents ($\mu\text{g. or units/ml.}$)					
	Novo ^d	Vanco ^e	Bac ^f	Risto ^g	Ery ^h	Oleano ⁱ
Reiter ^a	+(4)	1*	0.3	4	0.3	+(2)
Kazan ^a	+(4)	1	0.3	4	0.3	+(2)
<i>T. refringens</i>	+(4)	1	0.3	1	0.3	0.7
Calligyrum ^b	+(4)	0.7	0.3	1	0.3	+(2)
Nichols ^b	+(4)	0.7	0.3	1	0.3	2
Microdentium FM ^c	+(4)	1	0.7	1	0.3	+(2)
Ambiguum ^c	+(4)	0.7	0.3	1	0.3	+(2)
T-32-A ^c	4	4	0.3	0.7	0.3	0.7
<i>T. vincentii</i> N-9	1	1	0.3	+(4)	0.3	+(2)
HO 5	+(4)	+(4)	0.7	+(4)	0.3	+(2)
HO 18	+(4)	4	0.3	4	0.3	+(2)
HO 27	+(4)	4	0.3	+(4)	0.3	+(2)
PF 23	1	+(4)	1	+(4)	0.3	0.7
PF 31	4	+(4)	0.7	+(4)	0.3	+(2)
PF 39	0.7	+(4)	0.7	+(4)	0.3	2
PF 28	0.7	+(4)	0.7	+(4)	0.3	+(2)
PF 44	4	+(4)	1	+(4)	0.3	+(2)
DP-1	+(7.5)	7.5	0.5	+(0.5)	0.5	ND
DP-2	7.5	7.5	+(2.5)	+(0.5)	0.5	ND

^a*T. phagedenis*, ^b*T. refringens*, ^c*T. denticola*, ^dnovobiocin, ^evancomycin, ^fbacitracin, ^gristocetin, ^herythromycin, ⁱoleandomycin, ND = not done.

*concentration at which no growth appeared in 3 days. + = growth at concentration in parentheses

TABLE III Growth inhibition of treponemes by tetracyclines and chloramphenicol using the tube-disc method

Treponeme strains	Inhibitory concentrations of antimicrobial agents ($\mu\text{g./ml.}$)					
	Tet ^d	Chlortet ^e	Oxyt ^f	Meth ^g	Demeth ^h	Chloram ⁱ
Reiter ^a	0.7*	4	0.7	0.7	0.7	+(4)
Kazan ^a	4	4	0.7	0.7	0.7	+(4)
<i>T. refringens</i>	0.7	4	0.7	0.7	0.7	+(4)
Calligyrum ^b	0.7	4	4	0.7	1	+(4)
Nichols ^b	0.7	1	0.7	0.7	0.7	+(4)
Microdentium FM ^c	1	4	1	0.7	1	+(4)
Ambiguum ^c	0.7	1	0.7	0.7	0.7	+(4)
T-32-A ^c	1	4	0.7	0.7	0.7	+(4)
<i>T. vincentii</i> N-9	+(4)	+(4)	+(4)	+(4)	+(4)	+(4)
HO 5	0.7	4	0.7	0.7	4	+(4)
HO 18	1	4	0.7	0.7	0.7	+(4)
HO 27	1	4	0.7	0.7	0.7	+(4)
PF 23	+(4)	+(4)	+(4)	+(4)	+(4)	+(4)
PF 31	+(4)	+(4)	+(4)	+(4)	+(4)	+(4)
PF 39	+(4)	+(4)	+(4)	+(4)	+(4)	+(4)
PF 28	+(4)	+(4)	+(4)	+(4)	+(4)	+(4)
PF 44	+(4)	+(4)	+(4)	+(4)	+(4)	+(4)
DP-1	7.5	7.5	ND	ND	ND	7.5
DP-2	+(7.5)	+(7.5)	ND	ND	ND	7.5

^a*T. phagedenis*, ^b*T. refringens*, ^c*T. denticola*, ^dtetracycline, ^echlortetracycline, ^foxytetracycline, ^gmethacycline, ^hdemethylchlortetracycline, ⁱchloramphenicol, ND = not done. *concentration at which no growth appeared in 3 days. + = growth at concentration in parentheses

All strains grew in medium containing 40 $\mu\text{g./ml.}$ thiosulphil, trisulphapyrimidines, sulphadiazine, sulphamerazine, sulphamethoxypyridazine, sulphathiazole, sulphasomidine, or sulphafurazole. They all grew in medium containing lincomycin (0.3 to 0.5 $\mu\text{g./ml.}$), viomycin (1 to 2.5 $\mu\text{g./ml.}$), colistin sulphate (1 $\mu\text{g./ml.}$), Polymyxin B sulphate (40 to 75 units/ml.), nitrofurantoin (40 to 75 $\mu\text{g./ml.}$), nalidixic acid (4 to 15 $\mu\text{g./ml.}$), methenamine mandelate (400 $\mu\text{g./ml.}$), and mycostatin (14 $\mu\text{g./ml.}$). Most treponemes grew in medium with oxacillin (0.1 to 0.5 $\mu\text{g./ml.}$), oleandomycin (2 $\mu\text{g./ml.}$), chloramphenicol

(4 $\mu\text{g./ml.}$), streptomycin (1 to 2.5 $\mu\text{g./ml.}$), dihydrostreptomycin (1 to 2.5 $\mu\text{g./ml.}$), kanamycin (4 $\mu\text{g./ml.}$), and neomycin (1 to 7.5 $\mu\text{g./ml.}$).

Discussion

Treponemes under study did not colonize on agar plates incubated in anaerobic jars. Colonial growth on pre-reduced agar media in prescription bottles was slow and difficult to see, so that zones of inhibition could not be readily observed. Failure to determine antibiotic sensitivity of treponemes by

TABLE IV Growth inhibition of treponemes by aminoglycoside and peptide antibiotics and triclobisium chloride

Treponeme strains	Inhibitory concentrations of antimicrobial agents ($\mu\text{g. or units/ml.}$)				
	<i>Strep</i> ^d	<i>DHSM</i> ^e	<i>Kan</i> ^f	<i>Neo</i> ^g	<i>Triclo</i> ^h
Reiter ^a	+(1)	+(1)	+(4)	+(1)	35
Kazan ^a	+(1)	+(1)	+(4)	+(1)	35
<i>T. refringens</i>	1*	1	+(4)	+(1)	35
Calligyrum ^b	+(1)	1	+(4)	+(1)	35
Nichols ^b	0.7	1	0.7	0.7	35
Microdentium FM ^c	+(1)	+(1)	+(4)	+(1)	+(140)
Ambiguum ^c	+(1)	+(1)	+(4)	+(1)	+(140)
T-32-A ^c	+(1)	+(1)	4	+(1)	+(140)
<i>T. vincentii</i> N-9	+(1)	+(1)	+(4)	+(1)	+(140)
HO 5	+(1)	+(1)	+(4)	+(1)	35
HO 18	+(1)	1	4	+(1)	35
HO 27	+(1)	+(1)	+(4)	+(1)	35
PF 23	+(1)	+(1)	+(4)	+(1)	35
PF 31	+(1)	+(1)	+(4)	+(1)	140
PF 39	+(1)	+(1)	+(4)	+(1)	35
PF 28	+(1)	+(1)	+(4)	+(1)	35
PF 44	+(1)	+(1)	+(4)	+(1)	140
DP-1	+(2.5)	+(2.5)	ND	+(7.5)	ND
DP-2	+(2.5)	+(2.5)	ND	7.5	ND

^a*T. phagedenis*, ^b*T. refringens*, ^c*T. denticola*, ^dstreptomycin, ^edihydrostreptomycin, ^fkanamycin, ^gneomycin, ^htriclobisium chloride, ND = not done. *concentration at which no growth appeared in 3 days. + = growth at concentration in parentheses

discs on solidified agar indicated that a different method was needed for rapid antibiotic sensitivity determination for spirochaetes. Since spirochaetes grow well and quickly in pre-reduced semi-solid media, and growth can be observed in 1 to 3 days, the addition of antibiotic discs to a known amount of pre-reduced media provided us with a rapid method of determining antibiotic sensitivity of spirochaetes.

Comparison of results of the disc-tube method with those of the tube-dilution method (Abramson and Smibert, 1971) show that both methods give very similar figures for inhibitory concentrations with most strains. The dilutions were not exactly the same with the two methods but inhibition of treponemes was usually in the same concentration range. For example, *T. refringens* was inhibited by 0.3 $\mu\text{g./ml.}$ penicillin G using the disc method and by 0.1 $\mu\text{g./ml.}$ using the tube-dilution method, while the Reiter strain of *T. phagedenis* was inhibited by 0.7 $\mu\text{g./ml.}$ tetracycline using the disc method and by 1 $\mu\text{g./ml.}$ using tube dilutions.

With a few antibiotics, however, the results differed with some strains. For example, *T. refringens* was inhibited by 0.7 $\mu\text{g./ml.}$ of oxytetracycline using discs but required 10 $\mu\text{g./ml.}$ using tube-dilutions, while strain HO-5 grew in 4 $\mu\text{g./ml.}$ of vancomycin using discs but was inhibited by 1 $\mu\text{g./ml.}$ in tube-dilution experiments. These results were repeated several times and no reason was found for these differences.

In the case of oxacillin, nafcillin, cloxacillin, and lincomycin, the discs available contained too little antibiotic (1-2 $\mu\text{g./disc}$) to produce inhibitory

concentrations in semi-solid media, even if several discs were used in each tube.

Rapid determination of the susceptibility of treponemes by an antibiotic disc method is preferable to the more tedious tube-dilution technique. When using the disc method it is recommended that discs containing a total of 30 $\mu\text{g.}$ antibiotic be placed into 7.5 ml. of pre-reduced culture medium for a final concentration of approximately 4 $\mu\text{g./ml.}$ Each tube should be inoculated with 0.3 ml. of a 24 to 48-hr treponeme culture and mixed to disperse the inoculum. In addition, one high-concentration disc of thiosulphil, trisulphapyrimides, sulphadiazine, sulphamerazine, sulphamethoxypyridazine, sulphathiazole, sulphisomidine, sulphisoxazole, nitrofurantoin, methenamine mandelate, or Polymyxin B sulphate should be placed in 7.5 ml. of a medium. Susceptibility to all antibiotic discs can be determined readily by the disc-tube method except for oxacillin, nafcillin, cloxacillin, and lincomycin. The final concentration of an antibiotic in the disc test should approximate to the levels of antibiotic that can be attained in the blood, and results of susceptibility testing can thus be of value clinically. In the disc test, if there is no growth in a pre-reduced medium in 3 days, the spirochaete can be considered to be susceptible to the antibiotic.

Summary

Seventeen strains of treponemes and two free-living *Spirochaeta* spp. isolated from a duck pond were tested for their susceptibility to 33 antimicrobial

agents by suspending antibiotic discs in a culture in semi-solid medium. The tube-disc method was found to be a useful rapid method for determining the antibiotic susceptibility of spirochaetes. Inhibitory concentrations of the tube-disc method compared well with results of the tube-dilution method.

Zones of inhibition were not observed in solidified media since the growth of spirochaetes is transparent and barely discernible so that standard disc methods could not be used for testing.

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Une méthode pour apprécier la sensibilité des spirochètes aux antibiotiques et utilisant des disques d'antibiotiques

SOMMAIRE

En déposant des disques d'antibiotiques sur une culture en milieu semi-solide, on a étudié la sensibilité à 33 agents antimicrobiens de 17 souches de tréponèmes et de deux *Spirocheta* spp isolés directement dans une mare à canards. Cette méthode sur culture en tube se montra être utile et rapide pour déterminer la sensibilité des spirochètes aux antibiotiques. Avec cette méthode, les concentrations inhibitrices se comparent bien avec les résultats obtenus par la méthode des dilutions en tubes.

Sur les milieux solides, les zones d'inhibition n'apparaissent pas car la pousse des spirochètes est transparente et à peine discernable; ceci fait que les méthodes standard avec les disques ne peuvent pas être utilisées.